Vandkvalitetsinstituttet
Water Quality Institute

# R E P O R T - D A T A S H E E T

TITLE: GROWTH INHIBITION TEST OF EZEFLO F78 SURFACTANT WITH SKELETONEMA COSTATUM (DIATOM)

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PROJECT MANAGER : GIP

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# REPORT

SKELETONEMA COSTATUM

MARINE ALGAL GROWTH INHIBITION TEST

WITH

SURFACTANT FO82

NOTOX Project 113232
NOTOX Substance 36765

STATEMENT OF GLP COMPLIANCE

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NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice,

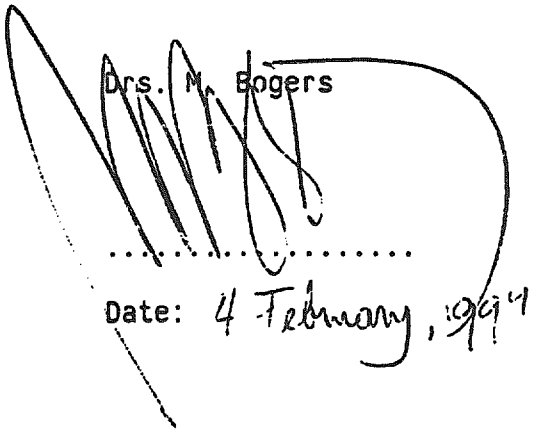
which are essentially in conformity with:

United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

United States Environmental Protection Agency, (FIFRA). Title 40 Code of Federal Regulations Part 160.

United States Environmental Protection Agency, (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director Drs. M. Bogers



Date: 4 February, 1994

## QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

Study procedures were subject to periodic inspections and general non study specific processes were also inspected at periodic intervals.

This report was audited by the NOTOX Quality Assurance Unit and the methods and results accurately reflect the raw data.

| Dates of Q.A.U. Inspections/Audits | Reporting Dates |
|---|---|
| 19-11-1993<br>08-12-1993<br>15-12-1993<br>03-02-1994 | 19-11-1993<br>08-12-1993<br>15-12-1993<br>03-02-1994 |

Quality Assurance Manager C.J. Mitchell B.Sc.

Date: 03-02-94

REPORT APPROVAL

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STUDY DIRECTOR: Drs. M. Bogers

.....

Date: 4 February, 1994.

MANAGEMENT: Ing. E.J. van de Waart
Section Head, Genetic &
Eco-Toxicology

.....

Date: 04/02/1994

## PREFACE

| | |
|---|---|
| Sponsor | Dowell Schlumberger<br>c/o. P.O. Box 20<br>4780 AA MOERDIJK<br>The Netherlands |
| Study Monitor | Mr. H. Romijn |
| Testing Facility | NOTOX B.V.<br>Hambakenwetering 3<br>5231 DD 's-Hertogenbosch<br>The Netherlands |
| Aquatic Toxicology:<br>Study Director | Drs. M. Bogers |
| Study Plan | Start : November 29, 1993<br>Completion : December 15, 1993 |

## TEST SUBSTANCE

| | |
|---|---|
| Identification | SURFACTANT FO82 |
| Description | White liquid, partly crystallized (determined at NOTOX) |
| Batch | 51493 |
| Purity | 100% |
| Instructions for test substance storage | At room temperature in the dark |
| Stability under storage conditions | Stable |
| Expiry date | June 01, 1994 |
| Stable for at least 4 hours in vehicle | Water: yes |

## PURPOSE

The purpose of the study was to evaluate the test substance for its ability to inhibit the growth of the marine algal species Skeletonema costatum in a short-term experiment.

## GUIDELINES

The study procedure described in this report is based on the following guidelines:

ISO (Draft) International Standard 10253: "Water Quality - Marine algal growth inhibition test with Skeletonema costatum and Phaeodactylum tricornutum on 11, August 1991.

In addition the study procedure is based on:
The Parcom Ring test protocol: "Technical support document for the ISO DP 10253 Standard Method".

OECD guideline for Testing of Chemicals, guideline No. 201: "Alga, Growth Inhibition Test", Adopted June 7, 1984, with some modifications.

EEC Directive 67/548 amended November 18, 1987 (87/302), OJEC L133 V31, Part C: Methods for the determination of ecotoxicity, "Algal Inhibition Test".

## ARCHIVING

NOTOX B.V. will archive the following data for at least 10 years: protocol, report, test substance reference sample, all specimens and raw data.

## DEFINITIONS

Cell density is the number of cells per millilitre.

Growth is the increase in cell density over the test period.

Growth rate is the increase in cell density per unit time.

EC50 is the concentration of test substance which results in a 50% reduction in either growth or growth rate relative to the control.

No Observed Effect Concentration (NOEC) is the highest tested concentration at which the measured parameter(s) show(s) no significant inhibition of growth relative to control values.

## TEST SYSTEM

| | |
|---|---|
| Species | Skeletonema costatum, strain: NIVA BAC1 |
| Reason for selection | This system is an unicellular algal species sensitive to toxic substances in the marine ecosystem and has been selected as an internationally accepted species. |
| Control of sensitivity | A reference test with potassium dichromate (Merck, Art. 4864) is carried out approximately every 3 months. The results of the most recent test are appended to this report. |

## RANGE-FINDING TEST

A range-finding test preceded the final test to provide information about the range of concentrations to be used in the final test. The range to which algae were exposed was 0.1 to 1000 mg/l, increasing by a factor 10.

## TEST PROCEDURES AND CONDITIONS

| | |
|---|---|
| Test type | Static |
| Test vessels | 100 ml, all-glass |
| Medium | ISO-medium formulated according to the International Standards "Water quality - Marine algal growth inhibition test" with Skeletonema costatum and Phaeodactylum tricornutum October, 1988 (formulated using natural seawater, in such a way that precipitation did not occur). The ISO-medium has the following composition |

| | |
|---|---|
| $FeCl_3.6H_2O$ | 140 ug/l (Fe) |
| $MnCl_2.4H_2O$ | 605 ug/l (Mn) |
| $ZnSO_4.7H_2O$ | 150 ug/l (Zn) |
| $CuSO_4.5H_2O$ | 0.6 ug/l (Cu) |
| $CoCl_2.6H_2O$ | 1.5 ug/l (Co) |
| $H_3BO_3$ | 17.1 mg/l |
| $Na_2EDTA$ | 15.0 mg/l |
| Thiamin hydrochloride | 25 ug/l |
| Biotin | 0.005 ug/l |
| $B_{12}$ | 0.05 ug/l |
| $K_3PO_4$ | 3.0 ug/l |
| $NaNO_3$ | 50 ug/l |
| $Na_2SiO_3.9H_2O$ | 19.4 ug/l |

| | |
|---|---|
| Cell density | An initial cell density of 1 x $10^4$ cells/ml using a 3 days old preculture with a cell density of 1.8 x $10^6$ cells/ml. |

| | |
|---|---|
| Test duration | 48 hours |
| Illumination | Continuously using TLD-lamps of 18 Watt (Philips, Spain), yielding 6000-6500 lux. |
| Temperature of the medium | 20 ± 2°C |
| Incubation | During incubation the algal cells were kept in suspension by continuous shaking. |
| Test concentrations | 0.056, 0.10, 0.18, 0.32, 0.56 and 1.0 mg/l. |
| Control | Test medium without test substance or other additives (blank). |
| Replicas | 3 replicas of each test concentration.<br>6 replicas of 0 mg/l. |

## PREPARATION OF TEST MEDIA

The test media were prepared using a stock solution in ISO-medium with a nominal concentration of 2 mg/l. Exact aliquots of this stock solution were diluted up to 100 ml of ISO-medium providing test substance concentrations of a factor 2 greater than the required concentrations. Subsequently, these solutions were mixed with ISO-medium containing 2 x $10^4$ algal cells/ml at a ratio of 1:1. Each vessel contained a final volume of 50 ml. At the start of the test all test solutions appeared clear without precipitation.

## MEASUREMENTS

| | |
|---|---|
| pH | At the beginning and at the end of the test. |
| Temperature of the medium | Every day in a control vessel with ISO-medium but without algae. |

## RECORDING OF CELL DENSITIES

At the beginning of the test cells were counted by microscope using a counting chamber. Thereafter cell densities were determined by spectrophotometric measurement of samples at 720 nm using a Lambda 1 Spectrophotometer (Perkin Elmer, Illinois, USA), with a cuvette of 5 cm path-length. Algal medium was used as blank.

## ACCEPTABILITY OF THE TEST

The cell density in the control cultures should have increased by a factor of at least 16 within the exposure period with a maximum of three days.

DATA HANDLING

Calibration curve:

At the end of the final test a calibration curve was made using dilutions of two of the negative control cultures. Cell density was plotted versus extinction using spectrophotometric measurements of a minimum of six dilutions with different cell densities. The calibration curve was composed using linear regression. The equation of this curve was then used to calculate the cell densities of the various test media at different points in time during the test period.

Comparison of areas under the growth curves:

The area below the growth curve was calculated using the formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

Where: A = area
$N_0$ = nominal number of cells/ml at the start of the test
$N_1$ = measured number of cells/ml at $t_1$
$N_n$ = measured number of cells/ml at $t_n$
$t_1$ = time of first measurements after beginning of the test
$t_n$ = time of $n^{th}$ measurement after beginning of the test

The percentage inhibition of cell growth at each test concentration ($I_T$) was calculated using the following formula:

$$I_T = \frac{A_C - A_T}{A_C} \times 100$$

Where: $A_C$ = area below the growth curve obtained in the control
$A_T$ = area below the growth curve at each test substance concentration

Growth inhibition was calculated for the total period of 48h.

Comparison of growth rates:

The average specific growth rate ($\mu$) for exponentially growing cultures was calculated as:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

The average growth rate at each test substance concentration was then compared to the control value and the percentage reduction in growth rate was calculated.

Determination of the NOEC and calculation of the EC50:

For determination of the NOEC and the EC50 the approaches recommended in the OECD guideline (201, adopted 7 June 1984) were used. An effect was considered to be significant if statistical analysis of the data obtained for the test concentrations compared with those obtained in the negative control revealed significant reduction of growth or inhibition of growth rate (Williams' test, TOXSTAT Release 3.0, September 1989, D.D. Gulley, A.M. Boelter, H.L. Bergman).
Calculation of the EC50 values was based on the probits of the percentages of growth inhibition and the percentages of growth rate reduction versus the logarithms of the corresponding concentrations of the test substance using the maximum likelihood estimation method (Finney, D.J., 1971: Probit analysis, Cambridge University Press, Cambridge, U.K., 3rd edition). For growth inhibition: the $E_BC50$ (0-48h) was calculated, and for growth rate reduction: the $E_RC50$ (0-48h) was calculated.

## RESULTS

### Range-finding test

In the range-finding test no growth inhibition was recorded at the test concentration of 0.1 mg/l, whereas total growth inhibition was recorded from 1 mg/l upwards.

### Final test: Mean cell densities

Table 1 shows mean cell densities measured at 24-hour intervals at the different concentrations of SURFACTANT FO82. The respective growth curves are shown in Figure 1 (see the Appendix I for the calibration curve, individual cell extinctions and cell densities).

### Final test: Inhibition of cell growth and reduction of growth rate

Table 2 shows the calculation of the percentage of inhibition of cell growth and the percentage of growth rate reduction at different time intervals. Statistical analysis of the data for areas under the growth curves (cell growth and growth rate) are shown in Appendix II.

Inhibition of cell growth increased with increasing concentration of SURFACTANT FO82 from 0.18 mg/l upwards, resulting in almost total inhibition at 1.0 mg/l. Statistically significant inhibition of cell growth was found at test concentrations of 0.32 mg/l and higher (Williams' test: $P=0.05$).

Growth rate reduction increased with increasing concentration of SURFACTANT FO82 from 0.32 mg/l upwards, resulting in 81% reduction at 1.0 mg/l. Statistically significant reduction of growth rate was found at test concentrations of 0.56 mg/l and higher (Williams' test: $P=0.05$).

### Final test: Experimental conditions

Table 3 shows the pH recorded at the beginning and the end of the test. Incidentally, pH increased slightly beyond one unit during the 48-hour test period.
The temperature of the test medium ranged from 19.9 to 21.0°C.

### Acceptability of the test

In the controls the cell density increased by a factor 107 within 2 days. Further, test conditions remained within the ranges prescribed by the protocol, except for an incidental increase of pH by more than 1 unit. However, the deviation was not more than 0.2 unit and related with normal algal growth.

Determination of EC50 values

Figure 2 and 3 show the curves for growth inhibition and growth rate reduction versus the log of the concentration. From these curves the EC50 values with the respective 95% fiducial limits have been calculated (see Tables 4 and 5).

## CONCLUSIONS

Under the conditions of the present study, SURFACTANT FO82 significantly inhibited cell growth of the marine algal species Skeletonema costatum at and above 0.32 mg/l, whereas significant growth rate reduction was recorded at concentrations of 0.56 mg/l and higher.

The EC50 for cell growth inhibition ($E_BC50$:0-48h) was 0.44 mg/l with a 95% fiducial limits ranging from 0.38 to 0.55 mg/l.

The EC50 for growth rate reduction ($E_RC50$:0-48h) was 0.70 mg/l with a 95% fiducial limits ranging from 0.65 to 0.75 mg/l.

The NOEC was 0.18 mg/l for cell growth inhibition ($NOE_BC$) and 0.32 mg/l for growth rate reduction ($NOE_RC$).

TABLE 1: Mean cell densities[1]

| Nominal concentration (mg/l) | Mean cell densities during exposure (x10$^4$ cells/ml) 0h | 24h | 48h |
|---|---|---|---|
| 0.00 | 1.00 | 12.68 | 107.1 |
| 0.056 | 1.00 | 17.97 | 125.8 |
| 0.10 | 1.00 | 16.10 | 133.6 |
| 0.18 | 1.00 | 15.47 | 117.3 |
| 0.32 | 1.00 | 8.68 | 96.2 |
| 0.56 | 1.00 | 3.82 | 29.7 |
| 1.0 | 1.00 | 1.22 | 2.4 |

[1] Values are rounded off by the program used for calculations

TABLE 2: Percentage inhibition of cell growth and percentage reduction of growth rate.

| Nominal concentration (mg/l) | Cell growth (0-48h): Area (A) mean | Inhibition (%) | Growth rate (cells/ml/h[1]): 0-48h mean $\mu^1$= | Reduction (%) |
|---|---|---|---|---|
| 0.00 | 1553.86 | | 0.09732 | |
| 0.056 | 1905.50 | -22.6 | 0.10063 | -3.4 |
| 0.10 | 1954.04 | -25.8 | 0.10190 | -4.7 |
| 0.18 | 1743.12 | -12.2 | 0.09913 | -1.9 |
| 0.32 | 1326.47 | 14.6 | 0.09511 | 2.3 |
| 0.56 | 411.68 | 73.5 | 0.06887 | 29.2 |
| 1.0 | 22.40 | 98.6 | 0.01833 | 81.2 |

[1] $\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$

TABLE 3: pH levels recorded during the final study.

| Nominal concentration (mg/l) | Vessel | pH-values 0h | 48h |
|---|---|---|---|
| 0.00 | 1 | 8.3 | 9.1 |
| 0.056 | 1 | 7.9 | 9.0 |
| 0.10 | 1 | 8.0 | 9.2 |
| 0.18 | 1 | 8.1 | 9.2 |
| 0.32 | 1 | 8.1 | 9.1 |
| 0.56 | 1 | 8.1 | 8.4 |
| 1.0 | 1 | 8.2 | 8.3 |

FIGURE 1: Growth curves at different concentrations of SURFACTANT FO82

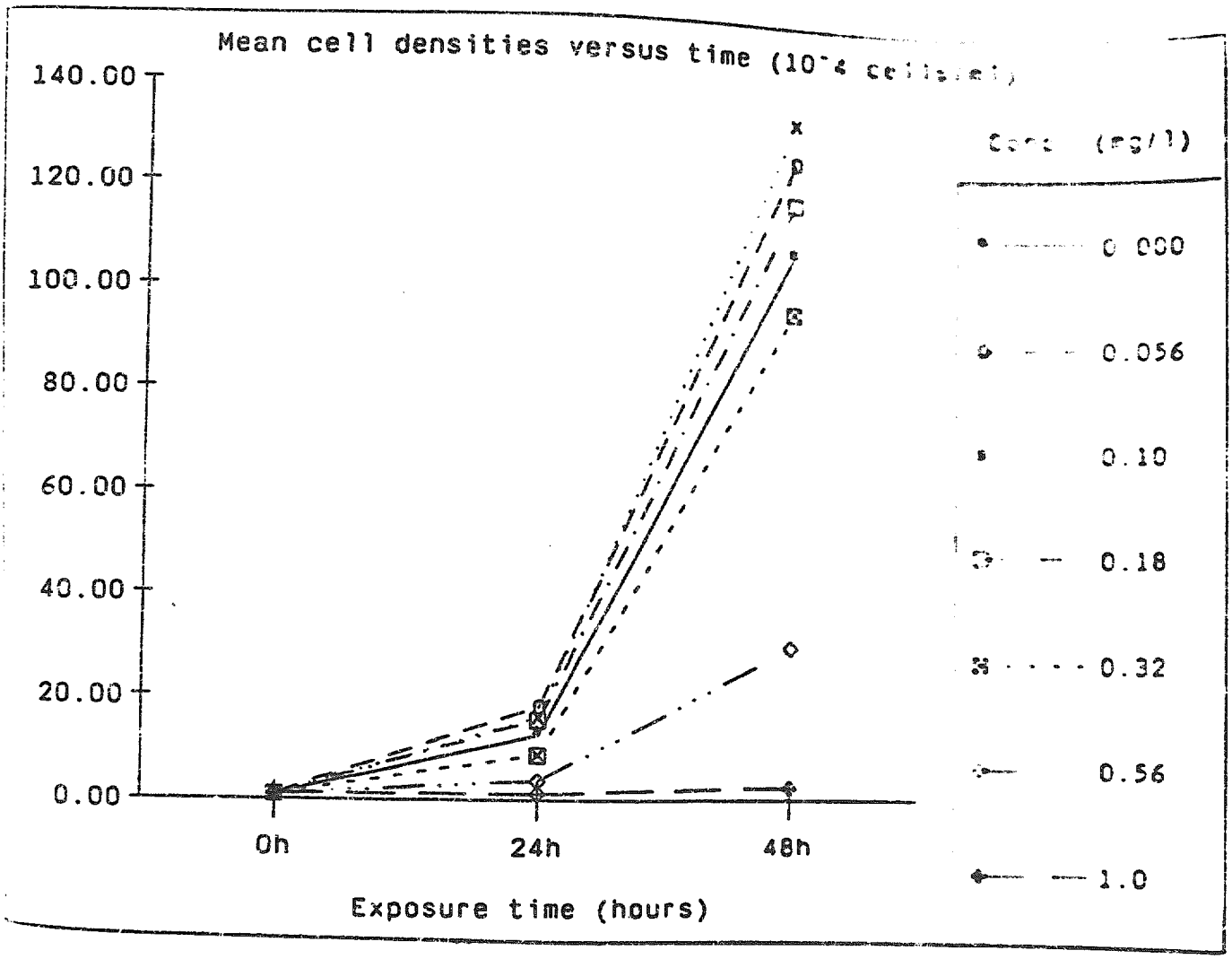


TABLE 4: EC50 Growth inhibition:

$EC_B50$ (0-48h)= 0.44 mg/l; 95% fiducial limits: 0.38 - 0.55 mg/l

heterogeneous data, h=1.62
index of regression significance: g=0.04
chi-squared=3.24, with 2 degrees of freedom
regression line: log10(conc.)=0.26+(probit-2.81)/5.71

| conc. mg/l | group size | response | corrected fraction | conc. metameter | working probit | chi2 |
|---|---|---|---|---|---|---|
| 0.18 | 100 | 0 | 0.00 | 0.26 | 2.79 | 0.00 |
| 0.32 | 100 | 15 | 0.15 | 0.51 | 4.22 | 2.63 |
| 0.56 | 100 | 74 | 0.74 | 0.75 | 5.61 | 0.07 |
| 1.00 | 100 | 99 | 0.99 | 1.00 | 7.05 | 0.54 |
| | | | | | | 3.24 |

FIGURE 2: Percentage inhibition of cell growth as function of log concentration (mg/l) of SURFACTANT FO82.

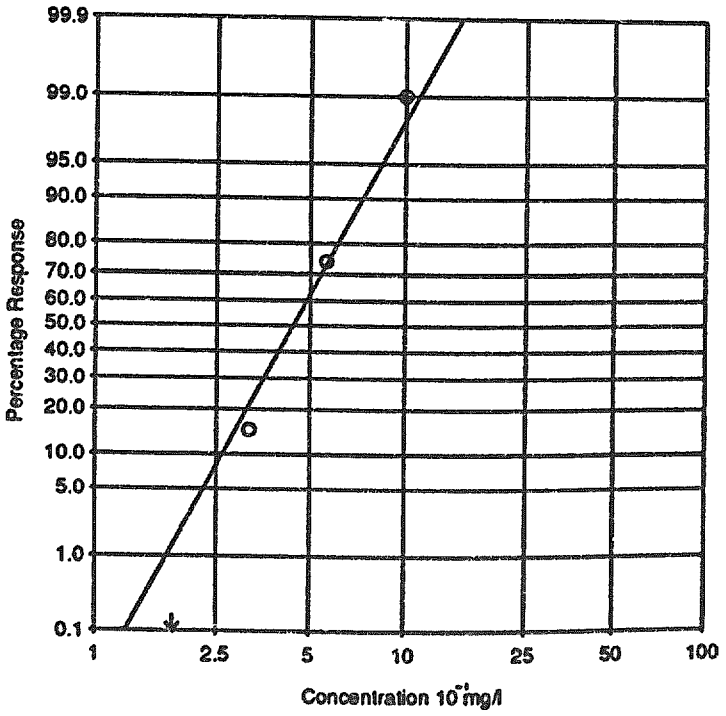


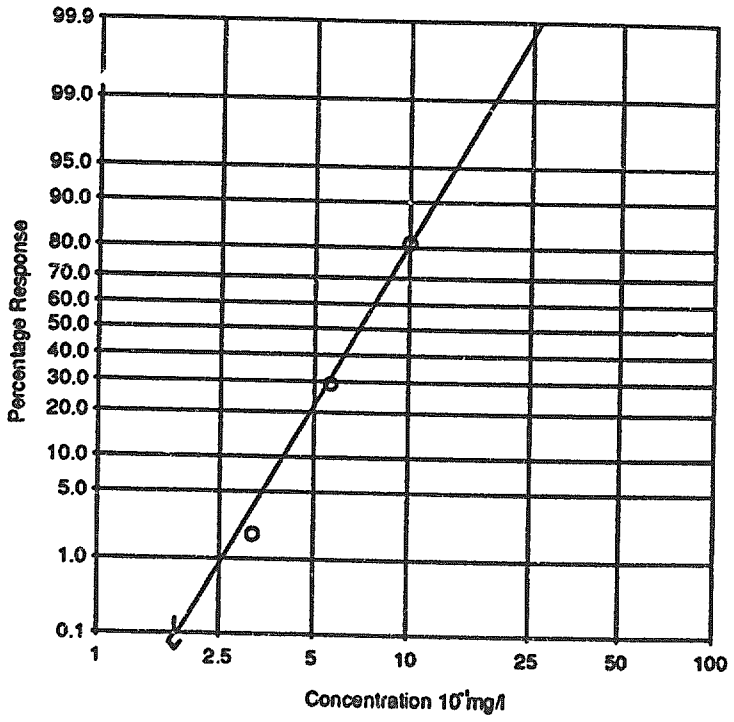
TABLE 5: EC50 Growth rate reduction:

$EC_R50$ (0-48h)= 0.70 mg/l; 95% fiducial limits: 0.65 - 0.75 mg/l

index of regression significance: g=0.03
chi-squared=0.32, with 1 degrees of freedom
regression line: log10(conc.)=0.77+(probit-4.61)/5.34

| conc. mg/l | group size | response | corrected fraction | expected fraction | chi2 |
|---|---|---|---|---|---|
| 0.32 | 100 | 2 | 0.02 | 0.02 | 0.10 |
| 0.56 | 100 | 29 | 0.29 | 0.31 | 0.17 |
| 1.00 | 100 | 81 | 0.81 | 0.80 | 0.05 |
| | | | | | 0.32 |

FIGURE 3: Percentage reduction of growth rate as function of log concentration (mg/l) of SURFACTANT FO82.



REFERENCE TEST

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NOTOX PCSK-7

Skeletonema costatum, marine algal growth inhibition test with potassium dichromate.

December, 1993.

This reference test was carried out to check the sensitivity of the test system used by NOTOX to potassium dichromate (Merck, Art. 4864, Batch 148 k15548364).

Concentrations : 0.56, 1.0, 1.8, 3.2 and 5.6 mg/l.

The EC50 for growth inhibition should be between 1 and 3.2 mg/l. (based on ISO Ring Test Document TC N 137).

Results: The nominal 48-hour EC50 for growth inhibition ($E_BC50$:0-48h) was 2.53 mg/l (95% confidence interval of 2.06 - 3.61 mg/l).

The raw data and report of this study are kept in the NOTOX archives. The test was performed under GLP conditions with a QA-check.

# APPENDIX I

## WORKSHEET DATA

TABLE I1: Calculation of the calibration curve:

| x | y | Calculated y |
|---|---|---|
| 0.80 | 10.5 | 6.76 |
| 1.17 | 11.5 | 9.35 |
| 2.34 | 18.0 | 17.45 |
| 4.68 | 30.0 | 33.65 |
| 11.7 | 83.5 | 82.24 |
| 23.4 | 156.5 | 163.23 |
| 46.8 | 327.5 | 325.20 |
| 117 | 811.5 | 811.11 |

x= cell density ($\times 10^4$ cells/ml)
y= extinction ($\times 10^{-3}$)
intercept = 1.26
r = 0.9999
slope = 6.9219
n = 8
Extinction at $10^4$ cells/ml: 8.18

TABLE I2: Individual values for extinction ($\times 10^{-3}$)

| Concentration mg/l | Vessel nr. | Hours of exposure 24h | 48h |
|---|---|---|---|
| 0.000 | 1 | 85 | 790 |
| | 2 | 96 | 698 |
| | 3 | 100 | 807 |
| | 4 | 78 | 699 |
| | 5 | 73 | 677 |
| | 6 | 102 | 786 |
| 0.056 | 1 | 120 | 995 |
| | 2 | 130 | 799 |
| | 3 | 127 | 823 |
| 0.10 | 1 | 116 | 810 |
| | 2 | 117 | 985 |
| | 3 | 105 | 984 |
| 0.18 | 1 | 104 | 939 |
| | 2 | 112 | 788 |
| | 3 | 109 | 713 |
| 0.32 | 1 | 64 | 666 |
| | 2 | 62 | 701 |
| | 3 | 58 | 634 |
| 0.56 | 1 | 22 | 108 |
| | 2 | 34 | 300 |
| | 3 | 27 | 212 |
| 1.0 | 1 | 7 | 19 |
| | 2 | 9 | 16 |
| | 3 | 12 | 19 |

# APPENDIX I continued

## WORKSHEET DATA

TABLE I3: Cell densities calculated from the individual extinction values
Number of ented cells at t=0 = $10^4$ cells/ml

| Concentration mg/l | Vessel nr. | Hours of exposure 0h | 24h | 48h |
|---|---|---|---|---|
| 0.000 | 1 | 1.00 | 12.10 | 113.9 |
| | 2 | 1.00 | 13.69 | 100.7 |
| | 3 | 1.00 | 14.27 | 116.4 |
| | 4 | 1.00 | 11.09 | 100.8 |
| | 5 | 1.00 | 10.36 | 97.6 |
| | 6 | 1.00 | 14.55 | 113.4 |
| 0.056 | 1 | 1.00 | 17.15 | 143.6 |
| | 2 | 1.00 | 18.60 | 115.3 |
| | 3 | 1.00 | 18.17 | 118.7 |
| 0.10 | 1 | 1.00 | 16.58 | 116.8 |
| | 2 | 1.00 | 16.72 | 142.1 |
| | 3 | 1.00 | 14.99 | 142.0 |
| 0.18 | 1 | 1.00 | 14.84 | 135.5 |
| | 2 | 1.00 | 16.00 | 113.7 |
| | 3 | 1.00 | 15.57 | 102.8 |
| 0.32 | 1 | 1.00 | 9.06 | 96.0 |
| | 2 | 1.00 | 8.78 | 101.1 |
| | 3 | 1.00 | 8.20 | 91.4 |
| 0.56 | 1 | 1.00 | 3.00 | 15.4 |
| | 2 | 1.00 | 4.73 | 43.2 |
| | 3 | 1.00 | 3.72 | 30.4 |
| 1.0 | 1 | 1.00 | 1.00 | 2.6 |
| | 2 | 1.00 | 1.12 | 2.1 |
| | 3 | 1.00 | 1.55 | 2.6 |

TABLE I4: Calculation of growth (area under growth curve) and growth rate per replica

| Concentration mg/l | Vessel nr. | Area (A) 0-48h | Growth rate 0-48h |
|---|---|---|---|
| 0.000 | 1 | 1621.76 | 0.09866 |
| | 2 | 1500.41 | 0.09608 |
| | 3 | 1703.24 | 0.09911 |
| | 4 | 1439.73 | 0.09611 |
| | 5 | 1384.25 | 0.09544 |
| | 6 | 1673.77 | 0.09856 |
| 0.056 | 1 | 2098.51 | 0.10347 |
| | 2 | 1793.39 | 0.09890 |
| | 3 | 1824.60 | 0.09952 |
| 0.10 | 1 | 1763.92 | 0.09918 |
| | 2 | 2070.78 | 0.10326 |
| | 3 | 2027.43 | 0.10324 |
| 0.18 | 1 | 1945.95 | 0.10227 |
| | 2 | 1711.91 | 0.09861 |
| | 3 | 1571.49 | 0.09652 |
| 0.32 | 1 | 1333.98 | 0.09510 |
| | 2 | 1387.72 | 0.09617 |
| | 3 | 1257.70 | 0.09407 |
| 0.56 | 1 | 220.98 | 0.05699 |
| | 2 | 595.45 | 0.07844 |
| | 3 | 418.62 | 0.07117 |
| 1.0 | 1 | 18.76 | 0.01961 |
| | 2 | 16.41 | 0.01575 |
| | 3 | 32.02 | 0.01961 |

# APPENDIX II

## STATISTICS: CELL GROWTH (0-48 HOURS)

Transform: NO TRANSFORMATION

Shapiro Wilks test for normality

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D = 347915.516
W = 0.953

Critical W (P = 0.05) (n = 24) = 0.916
Critical W (P = 0.01) (n = 24) = 0.884

---

Data PASS normality test at P=0.01 level. Continue analysis.

Bartletts test for homogeneity of variance

---

Calculated B statistic = 12.44
Table Chi-square value = 16.81 (alpha = 0.01)
Table Chi-square value = 12.59 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 2.43
Used for Chi-square table value ==> df (#groups-1) = 6

---

Data PASS homogeneity test at 0.01 level. Continue analysis.
NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

| GROUP | IDENTIFICATION | N | ORIGINAL MEAN | TRANSFORMED MEAN | ISOTONIZED MEAN |
|---|---|---|---|---|---|
| 1 | 0.00 | 6 | 1553.860 | 1553.860 | 1742.076 |
| 2 | 0.056 | 3 | 1905.500 | 1905.500 | 1742.076 |
| 3 | 0.10 | 3 | 1954.043 | 1954.043 | 1742.076 |
| 4 | 0.18 | 3 | 1743.117 | 1743.117 | 1742.076 |
| 5 | 0.32 | 3 | 1326.467 | 1326.467 | 1326.467 |
| 6 | 0.56 | 3 | 411.683 | 411.683 | 411.683 |
| 7 | 1.00 | 3 | 22.397 | 22.397 | 22.397 |

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

| IDENTIFICATION | ISOTONIZED MEAN | CALC. WILLIAMS | SIG P=.05 | TABLE WILLIAMS | DEGREES OF FREEDOM |
|---|---|---|---|---|---|
| 0.00 | 1742.076 | | | | |
| 0.056 | 1742.076 | 1.861 | *1 | 1.74 | k= 1, v=17 |
| 0.10 | 1742.076 | 1.861 | *1 | 1.82 | k= 2, v=17 |
| 0.18 | 1742.076 | 1.861 | *1 | 1.85 | k= 3, v=17 |
| 0.32 | 1326.467 | 2.248 | *2 | 1.87 | k= 4, v=17 |
| 0.56 | 411.683 | 11.291 | *2 | 1.87 | k= 5, v=17 |
| 1.00 | 22.397 | 15.139 | *2 | 1.88 | k= 6, v=17 |

[1] Statistically significant increase of cell growth
[2] Statistically significant inhibition of cell growth
s = 143.058
Note: df used for table values are approximate when v > 20.

# APPENDIX II

## STATISTICS: GROWTH RATE (0-48 HOURS)

Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

| INTERVAL | <-1.5 | -1.5 to <-0.5 | -0.5 to 0.5 | >0.5 to 1.5 | >1.5 |
|---|---|---|---|---|---|
| EXPECTED | 1.608 | 5.808 | 9.168 | 5.808 | 1.608 |
| OBSERVED | 0 | 9 | 4 | 11 | 0 |

Calculated Chi-Square goodness of fit test statistic = 12.9248
Table Chi-Square value (alpha = 0.01) = 13.277
Data PASS normality test. Continue analysis.

Hartley test for homogeneity of variance

Calculated H statistic (max Var/min Var) = 107.93
Closest, conservative, Table H statistic = 1705.0 (alpha = 0.01)

Used for Table H ==> R (# groups) = 7, df (# reps-1) = 2
Actual values ==> R (# groups) = 7, df (# avg reps-1) = 2.69
(average df used)

Data PASS homogeneity test. Continue analysis.
NOTE: This test requires equal replicate sizes. If they are unequal but do not differ greatly, the Hartley test may still be used as an approximate test (average df are used).

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

| GROUP | IDENTIFICATION | N | ORIGINAL MEAN | TRANSFORMED MEAN | ISOTONIZED MEAN |
|---|---|---|---|---|---|
| 1 | 0.00 | 6 | 0.097 | 0.097 | 0.099 |
| 2 | 0.056 | 3 | 0.101 | 0.101 | 0.099 |
| 3 | 0.10 | 3 | 0.102 | 0.102 | 0.099 |
| 4 | 0.18 | 3 | 0.099 | 0.099 | 0.095 |
| 5 | 0.32 | 3 | 0.095 | 0.095 | 0.069 |
| 6 | 0.56 | 3 | 0.069 | 0.069 | 0.018 |
| 7 | 1.00 | 3 | 0.018 | 0.018 | 0.018 |

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

| IDENTIFICATION | ISOTONIZED MEAN | CALC. WILLIAMS | SIG P=.05 | TABLE WILLIAMS | DEGREES OF FREEDOM |
|---|---|---|---|---|---|
| 0.00 | 0.099 | | | | |
| 0.056 | 0.099 | 0.622 | | 1.94 | ** 1, 19 * |
| 0.10 | 0.099 | 0.622 | | 1.86 | ** 2, 19 * |
| 0.18 | 0.099 | 0.571 | | 1.85 | ** 3, 19 * |
| 0.32 | 0.095 | 0.700 | | 1.83 | ** 4, 19 * |
| 0.56 | 0.069 | 9.000 | * | 1.83 | ** 5, 19 * |
| 1.00 | 0.018 | 24.983 | * | 1.83 | ** 6, 19 * |

s = 0.004
Note: df used for table values are approximate when v > 20.

# REPORT

## SKELETONEMA COSTATUM

## MARINE ALGAL GROWTH INHIBITION TEST

## WITH

## HIGH TEMP ACID GELLING AGENT J476C

NOTOX Project 114694
NOTOX Substance 37269

HIGH TEMP ACID GELLING AGENT J476C NOTOX Project 114694

STATEMENT OF GLP COMPLIANCE

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NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice,

which are essentially in conformity with:

United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

United States Environmental Protection Agency, (FIFRA). Title 40 Code of Federal Regulations Part 160.

United States Environmental Protection Agency, (TSCA). Title 40 Code of Federal Regulations Part 792.

Stability of test concentrations in test medium is excluded from this statement.

Study Director Drs M. Bogers

.....

Date: 11 February, 1994

QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

Study procedures were subject to periodic inspections and general non study specific processes were also inspected at periodic intervals.

This report was audited by the NOTOX Quality Assurance Unit and the methods and results accurately reflect the raw data.

| Dates of Q.A.U. Inspections/Audits | Reporting Dates |
|---|---|
| 03-12-1993 | 03-12-1993 |
| 08-12-1993 | 08-12-1993 |
| 15-12-1993 | 15-12-1993 |
| 10-01-1994 | 10-01-1994 |
| 10-02-1994 | 10-02-1994 |

Quality Assurance Manager C.J. Mitchell B.Sc.

Date: 21-2-94

REPORT APPROVAL

STUDY DIRECTOR:

Drs. M. Bogers

.....

Date: 11 February, 1994

MANAGEMENT:

Ing. E.J. van de Waart
Section Head, Genetic &
Eco-Toxicology

i.A.

Dr. P.J.J.M. Weterings
Managing Director

.....

Date: 15 February 1994

PREFACE

| | |
|---|---|
| Sponsor | Dowell Schlumberger<br>c/o. P.O. Box 20<br>4780 AA MOERDIJK<br>The Netherlands |
| Study Monitor | Mr. H. Romijn |
| Testing Facility | NOTOX B.V.<br>Hambakenwetering 3<br>5231 DD 's-Hertogenbosch<br>The Netherlands |
| Aquatic Toxicology:<br>Study Director | Drs. M. Bogers |
| Study Plan | Start : 08 December, 1993<br>Completion : 05 February, 1994 |

TEST SUBSTANCE

| | |
|---|---|
| Identification | High temp acid gelling agent J476C |
| Description | Milky emulsion |
| Batch | GT 920623 |
| Purity | Treat as 100% |
| Instructions for test substance storage | At room temperature in the dark |
| Stability under storage conditions | Stable |
| Expiry date | July 01, 1994 |
| Stable for at least 4 hours in vehicle | Water: not indicated |

## PURPOSE

The purpose of the study was to evaluate the test substance for its ability to inhibit the growth of the marine algal species Skeletonema costatum in a short-term experiment.

## GUIDELINES

The study procedure described in this report is based on the following guidelines:

ISO (Draft) International Standard 10253: "Water Quality - Marine algal growth inhibition test with Skeletonema costatum and Phaeodactylum tricornutum on 11, August 1991.

In addition the study procedure is based on:
The Parcom Ring test protocol: "Technical support document for the ISO DP 10253 Standard Method".

OECD guideline for Testing of Chemicals, guideline No. 201: "Alga, Growth Inhibition Test", Adopted June 7, 1984, with some modifications.

EEC Directive 67/548 amended November 18, 1987 (87/302), OJEC L133 V31, Part C: Methods for the determination of ecotoxicity, "Algal Inhibition Test".

## ARCHIVING

NOTOX B.V. will archive the following data for at least 10 years: protocol, report, test substance reference sample, all specimens and raw data.

## DEFINITIONS

Cell density is the number of cells per millilitre.

Growth is the increase in cell density over the test period.

Growth rate is the increase in cell density per unit time.

EC50 is the concentration of test substance which results in a 50% reduction in either growth or growth rate relative to the control.

No Observed Effect Concentration (NOEC) is the highest tested concentration at which the measured parameter(s) show(s) no significant inhibition of growth relative to control values.

## TEST SYSTEM

| | |
|---|---|
| Species | Skeletonema costatum, strain: NIVA BAC1 |
| Reason for selection | This system is an unicellular algal species sensitive to toxic substances in the marine ecosystem and has been selected as an internationally accepted species. |
| Control of sensitivity | A reference test with potassium dichromate (Merck, Art. 4864) is carried out approximately every 3 months. The results of the most recent test are appended to this report. |

## RANGE-FINDING TEST

Two range-finding tests preceded the final test to provide information about the range of concentrations to be used in the final test. The range to which algae were exposed was 0.1 to 1000 mg/l in the first test and 0.01 to 100 mg/l in the second both increasing by a factor 10.

## TEST PROCEDURES AND CONDITIONS

| | |
|---|---|
| Test type | Static |
| Test vessels | 100 ml, all-glass |
| Medium | ISO-medium formulated according to the International Standards "Water quality - Marine algal growth inhibition test" with Skeletonema costatum and Phaeodactylum tricornutum October, 1988 (formulated using natural seawater, in such a way that precipitation did not occur). The ISO-medium has the following composition |

| | | |
|---|---|---|
| $FeCl_3.6H_2O$ | 140 | µg/l (Fe) |
| $MnCl_2.4H_2O$ | 605 | µg/l (Mn) |
| $ZnSO_4.7H_2O$ | 150 | µg/l (Zn) |
| $CuSO_4.5H_2O$ | 0.6 | µg/l (Cu) |
| $CoCl_2.6H_2O$ | 1.5 | µg/l (Co) |
| $H_3BO_3$ | 17.1 | mg/l |
| $Na_2EDTA$ | 15.0 | mg/l |
| Thiamin hydrochloride | 25 | µg/l |
| Biotin | 0.005 | µg/l |
| $B_{12}$ | 0.05 | µg/l |
| $K_3PO_4$ | 3.0 | µg/l |
| $NaNO_3$ | 50 | µg/l |
| $Na_2SiO_3.9H_2O$ | 19.4 | µg/l |

| | |
|---|---|
| Cell density | An initial cell density of $1 \times 10^4$ cells/ml using a 3 days old preculture with a cell density of $383.5 \times 10^4$ cells/ml. |

| | |
|---|---|
| Test duration | 48 hours |
| Illumination | Continuously using TLD-lamps of 18 Watt (Philips, Spain), yielding 6000-6500 lux. |
| Temperature of the medium | 20 ± 2°C |
| Incubation | During incubation the algal cells were kept in suspension by continuous shaking. |
| Test concentrations | 0.018, 0.032, 0.056, 0.10, 0.18, 0.32 and 0.56 mg/l. |
| Control | Test medium without test substance or other additives (blank). |
| Replicas | 3 replicas of each test concentration.<br>6 replicas of 0 mg/l. |

## PREPARATION OF TEST MEDIA

A stock solution was prepared in ISO-medium with a nominal concentration of 100 mg/l. A second stock solution was prepared containing a 1/50 dilution of the first stock providing a concentration of 2 mg/l. Exact aliquots of this stock solution were diluted up to 100 ml of ISO-medium providing test substance concentrations of a factor 2 greater than the required concentrations. Subsequently, these solutions were mixed with ISO-medium containing $2 \times 10^4$ algal cells/ml at a ratio of 1:1. Each vessel contained a final volume of 50 ml. At the start of the test all test solutions appeared clear without precipitation.

## MEASUREMENTS

| | |
|---|---|
| pH | At the beginning and at the end of the test. |
| Temperature of the medium | Every day in a control vessel with ISO-medium but without algae. |

## RECORDING OF CELL DENSITIES

At the beginning of the test cells were counted by microscope using a counting chamber. Thereafter cell densities were determined by spectrophotometric measurement of samples at 720 nm using a Lambda 1 Spectrophotometer (Perkin Elmer, Illinois, USA), with a cuvette of 5 cm path-length. Algal medium was used as blank.

ACCEPTABILITY OF THE TEST

The cell density in the control cultures should have increased by a factor of at least 16 within the exposure period with a maximum of three days.

DATA HANDLING

Calibration curve:
At the end of the final test a calibration curve was made using dilutions of two of the negative control cultures. Cell density was plotted versus extinction using spectrophotometric measurements of a minimum of six dilutions with different cell densities. The calibration curve was composed using linear regression. The equation of this curve was then used to calculate the cell densities of the various test media at different points in time during the test period.

Comparison of areas under the growth curves:
The area below the growth curve was calculated using the formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

Where: A = area
$N_0$ = nominal number of cells/ml at the start of the test
$N_1$ = measured number of cells/ml at $t_1$
$N_n$ = measured number of cells/ml at $t_n$
$t_1$ = time of first measurements after beginning of the test
$t_n$ = time of $n^{th}$ measurement after beginning of the test

The percentage inhibition of cell growth at each test concentration ($I_T$) was calculated using the following formula:

$$I_T = \frac{A_C - A_T}{A_C} \times 100$$

Where: $A_C$ = area below the growth curve obtained in the control
$A_T$ = area below the growth curve at each test substance concentration

Growth inhibition was calculated for the total period of 48 h.

Comparison of growth rates:
The average specific growth rate ($\mu$) for exponentially growing cultures was calculated as:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

The average growth rate at each test substance concentration was then compared to the control value and the percentage reduction in growth rate was calculated.

Determination of the NOEC and calculation of the EC50:

For determination of the NOEC and the EC50 the approaches recommended in the OECD guideline (201, adopted 7 June 1984) were used. An effect was considered to be significant if statistical analysis of the data obtained for the test concentrations compared with those obtained in the negative control revealed significant reduction of growth or inhibition of growth rate (Williams' test, TOXSTAT Release 3.0, September 1989, D.D. Gulley, A.M. Boelter, H.L. Bergman).

Determination of the EC50 values was based on:

a. the probits of the percentages of growth inhibition versus the logarithms of the corresponding concentrations of the test substance using the maximum likelihood estimation method (Finney, D.J., 1971: Probit analysis, Cambridge University Press, Cambridge, U.K., 3rd edition).

b. the percentages of growth rate reduction recorded at the corresponding concentrations with 95 % confidence limits expressed by the highest concentration inducing no significant reduction and the lowest concentration inducing 100 % reduction.

Range-finding test

In the first range-finding test total growth inhibition was recorded at test substance concentrations of 1.0 mg/l or higher. Significant inhibition was recorded at 0.10 mg/l. The results of the second range-finding test showed no growth inhibition at 0.01 mg/l, but confirmed the toxicity recorded at higher concentrations.

Final test: Mean cell densities

Table 1 shows mean cell densities measured at 24-hour intervals at the different concentrations of HIGH TEMP ACID GELLING AGENT J476C. The respective growth curves are shown in Figure 1 (see the appendix I for the calibration curve, individual cell extinctions and cell densities).

Final test: Inhibition of cell growth and reduction of growth rate

Table 2 shows the calculation of the percentage of inhibition of cell growth and the percentage of growth rate reduction. Statistical analysis of the data for areas under the growth curves (cell growth and growth rate) are shown in Appendix II.

Inhibition of cell growth increased with increasing concentration of HIGH TEMP ACID GELLING AGENT J476C from 0.056 mg/l upwards resulting in 100 % inhibition at nominally 0.56 mg/l. Statistically significant inhibition of cell growth was found at test concentrations of 0.18 mg/l and higher (Williams' test: P=0.05).

Growth rate reduction increased with increasing concentration of HIGH TEMP ACID GELLING AGENT J476C from 0.10 mg/l upwards resulting in 100 % reduction at nominally 0.56 mg/l. Statistically significant reduction of growth rate was found at test concentrations of 0.18 mg/l and higher (Williams' test: P=0.05).

Final test: Experimental conditions

Table 3 shows the pH recorded at the beginning and the end of the test The test was performed in an incubator at a constant test medium temperature of 22 °C.

Acceptability of the test

In the controls the cell density increased by a factor 81 within 2 days. Further, all test conditions remained within the ranges prescribed by the protocol.

Determination of EC50 values

Figures 2 and 3 show the curves for growth inhibition and growth rate reduction versus the log of the concentration. From the curve in Figure 2, the EC50 value for growth inhibition has been calculated including the respective 95% confidence interval (see Table 4).

## CONCLUSIONS

Under the conditions of the present study, HIGH TEMP ACID GELLING AGENT J476C did not significantly inhibit cell growth or reduce growth rate of Skeletonema costatum at concentrations up to and including 0.10 mg/l (NOEC).

The EC50 for cell growth inhibition ($E_BC50$:0-48h) was 0.15 mg/l with a 95 % confidence interval ranging from 0.14 to 0.17 mg/l.

The EC50 for growth rate reduction ($E_RC50$:0-48h) was 0.32 mg/l with a 95 % confidence interval ranging from 0.10 (EC0) to 0.56 mg/l (EC100).

FIGURE 1: Growth curves at different concentrations of HIGH TEMP ACID GELLING AGENT J476C.

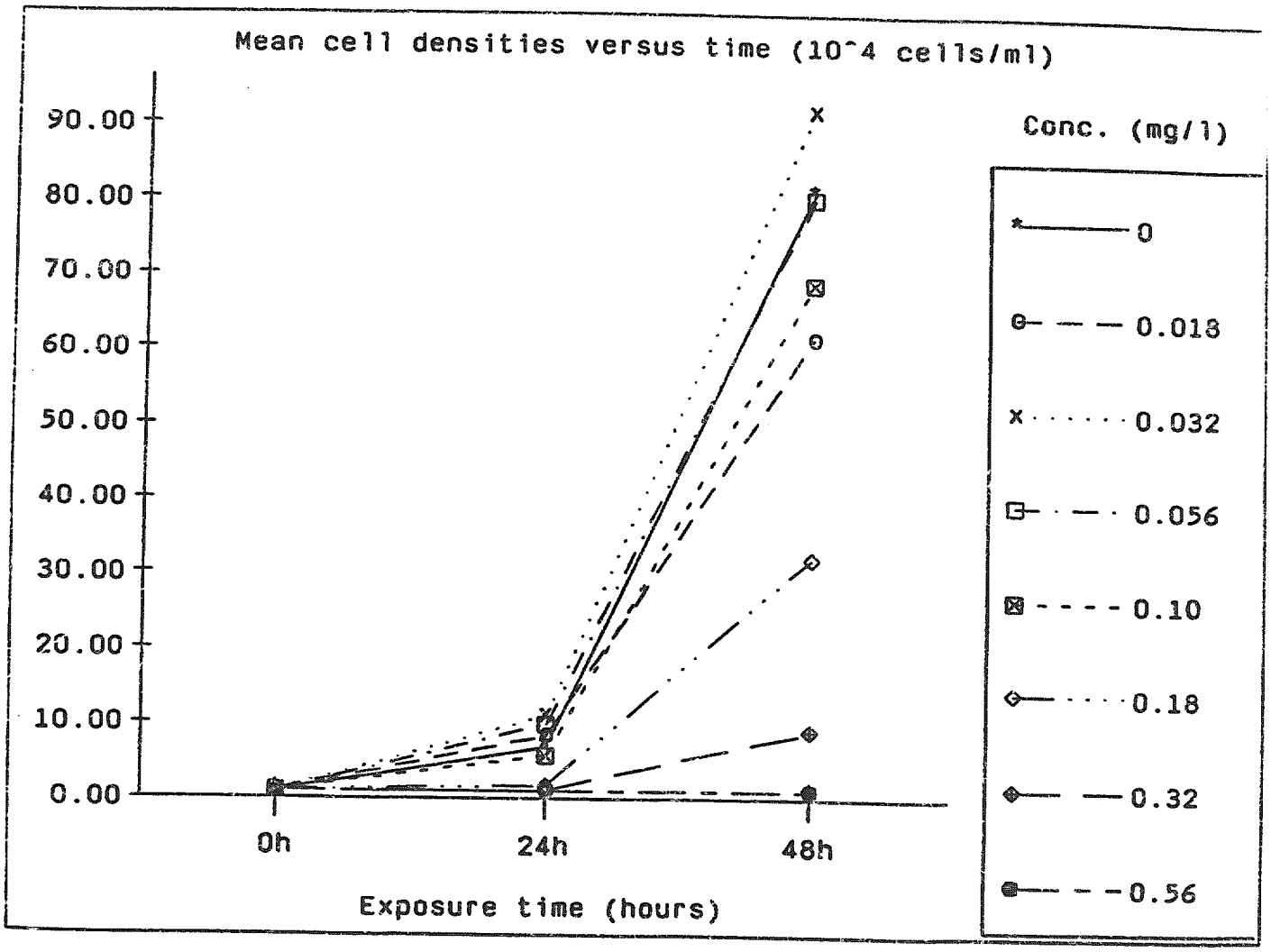


TABLE 4: EC50 Growth inhibition:

$E_BC50$= 0.15 mg/l (154 µg/l)
95 % fiducial limits: 0.14 - 0.17 mg/l (144.6 - 166.3 µg/l)

index of regression significance: g=0.00
chi-squared=2.03, with 3 degrees of freedom
regression line: log10(conc.)=1.75+(probit-3.05)/4.46

| conc. | group size | response | corrected fraction | expected fraction | chi2 |
|---|---|---|---|---|---|
| 56 | 100 | 0 | 0.00 | 0.00 | 0.00 |
| 100 | 100 | 16 | 0.16 | 0.20 | 1.01 |
| 180 | 100 | 64 | 0.64 | 0.62 | 0.20 |
| 320 | 100 | 91 | 0.91 | 0.92 | 0.19 |
| 560 | 100 | 100 | 1.00 | 0.99 | 0.62 |
| | | | | | 2.03 |

FIGURE 2: Percentage inhibition of cell growth as function of log concentration (µg/l) of HIGH TEMP ACID GELLING AGENT J476C.

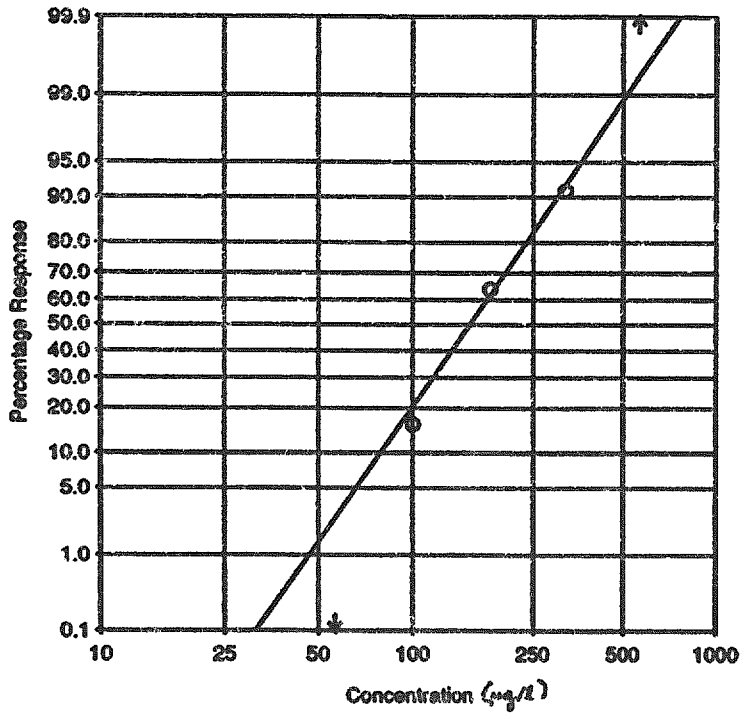
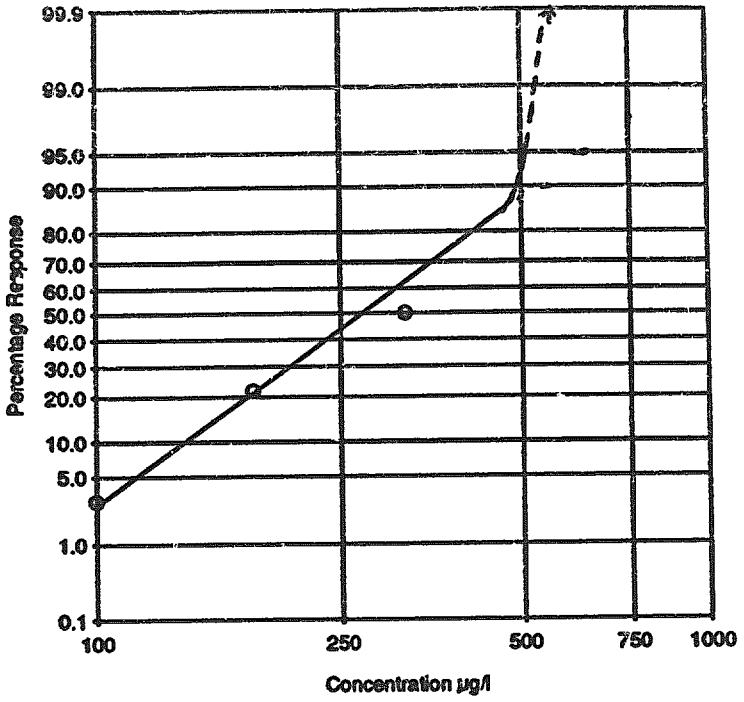


FIGURE 3: Percentage reduction of growth rate as function of log concentration (µg/l) of HIGH TEMP ACID GELLING AGENT J476C.



NOTOX PCSK-7

Skeletonema costatum, marine algal growth inhibition test with potassium dichromate.

December, 1993.

This reference test was carried out to check the sensitivity of the test system used by NOTOX to potassium dichromate (Merck, Art. 4864, Batch 148 k15548364).

Concentrations : 0.56, 1.0, 1.8, 3.2 and 5.6 mg/l.

The EC50 for growth inhibition should be between 1 and 3.2 mg/l. (based on ISO Ring Test Document TC N 137).

Results: The nominal 48-hour EC50 for growth inhibition ($E_BC50$:0-48h) was 2.53 mg/l (95% confidence interval of 2.06 - 3.61 mg/l).

The raw data and report of this study are kept in the NOTOX archives. The test was performed under GLP conditions with a QA-check.

# APPENDIX I

## WORKSHEET DATA

Table I1: Calculation of the calibration curve:

| x | y | Calculated y |
|---|---|---|
| 0.96 | 15 | 23.46 |
| 3.80 | 33 | 39.67 |
| 9.60 | 71 | 72.79 |
| 19 | 132 | 126.46 |
| 38 | 250 | 234.94 |
| 77 | 472 | 457.60 |
| 96 | 550 | 566.08 |

x= cell density (x10^4 cells/ml)
y= extinction (x 10^-3)
intercept = 17.57
r = 0.9985
slope = 5.7231
n = 7.00
Extinction at 1.0 x 10^4 cells/ml: 23.30

Table I2: Individual values for extinction (x10^-3)

| Concentration mg/l | Vessel nr. | Hours of exposure 24h | 48h |
|---|---|---|---|
| 0.000 | 1 | 51 | 245 |
| | 2 | 56 | 477 |
| | 3 | 58 | 524 |
| | 4 | 59 | 578 |
| | 5 | 58 | 530 |
| | 6 | 60 | 523 |
| 0.018 | 1 | 90 | 575 |
| | 2 | 83 | 506 |
| | 3 * | | |
| 0.032 | 1 | 85 | 573 |
| | 2 | 77 | 504 |
| | 3 | 80 | 550 |
| 0.056 | 1 | 74 | 515 |
| | 2 | 72 | 426 |
| | 3 | 78 | 483 |
| 0.10 | 1 | 45 | 400 |
| | 2 | 60 | 418 |
| | 3 | 46 | 412 |
| 0.18 | 1 | 32 | 247 |
| | 2 | 28 | 236 |
| | 3 | 12 | 119 |
| 0.32 | 1 | 10 | 80 |
| | 2 | 11 | 65 |
| | 3 | 12 | 61 |
| 0.56 | 1 | 8 | 20 |
| | 2 | 7 | 14 |
| | 3 | 10 | 14 |

* Vessel was lost due to a technical failure.

# APPENDIX I

## WORKSHEET DATA

Table I3: Cell densities calculated from the individual extinction values
Number of ented cells at t=0 = 1.0 x 10^4 cells/ml

| Concentration mg/l | Vessel nr. | Hours of exposure 0h | 24h | 48h |
|---|---|---|---|---|
| 0.000 | 1 | 1.00 | 5.84 | 39.7 |
| | 2 | 1.00 | 6.71 | 80.3 |
| | 3 | 1.00 | 7.06 | 88.5 |
| | 4 | 1.00 | 7.24 | 97.9 |
| | 5 | 1.00 | 7.06 | 89.5 |
| | 6 | 1.00 | 7.41 | 88.3 |
| 0.018 | 1 | 1.00 | 12.66 | 97.4 |
| | 2 | 1.00 | 11.43 | 85.3 |
| 0.032 | 1 | 1.00 | 11.78 | 97.1 |
| | 2 | 1.00 | 10.38 | 85.0 |
| | 3 | 1.00 | 10.91 | 93.0 |
| 0.056 | 1 | 1.00 | 9.86 | 86.9 |
| | 2 | 1.00 | 9.51 | 71.4 |
| | 3 | 1.00 | 10.56 | 81.3 |
| 0.10 | 1 | 1.00 | 4.79 | 66.8 |
| | 2 | 1.00 | 7.41 | 70.0 |
| | 3 | 1.00 | 4.97 | 68.9 |
| 0.18 | 1 | 1.00 | 2.52 | 40.1 |
| | 2 | 1.00 | 1.82 | 38.2 |
| | 3 | 1.00 | 1.00 | 17.7 |
| 0.32 | 1 | 1.00 | 1.00 | 10.9 |
| | 2 | 1.00 | 1.00 | 8.3 |
| | 3 | 1.00 | 1.00 | 7.6 |
| 0.56 | 1 | 1.00 | 1.00 | 1.0 |
| | 2 | 1.00 | 1.00 | 1.0 |
| | 3 | 1.00 | 1.00 | 1.0 |

Table I4: Calculation of growth (area under growth curve) and growth rate

| Concentration mg/l | Vessel nr. | Area (A) 0-48h | Growth rate 0-48h |
|---|---|---|---|
| 0.00 | 1 | 581.04 | 0.07672 |
| | 2 | 1088.46 | 0.09136 |
| | 3 | 1195.40 | 0.09339 |
| | 4 | 1312.82 | 0.09550 |
| | 5 | 1207.98 | 0.09364 |
| | 6 | 1201.69 | 0.09335 |
| 0.018 | 1 | 1436.53 | 0.09539 |
| | 2 | 1262.49 | 0.09264 |
| 0.032 | 1 | 1411.37 | 0.09532 |
| | 2 | 1233.14 | 0.09255 |
| | 3 | 1342.17 | 0.09444 |
| 0.056 | 1 | 1243.62 | 0.09302 |
| | 2 | 1048.62 | 0.08891 |
| | 3 | 1193.30 | 0.09163 |
| 0.10 | 1 | 880.88 | 0.08754 |
| | 2 | 981.53 | 0.08850 |
| | 3 | 910.24 | 0.08819 |
| 0.18 | 1 | 505.56 | 0.07690 |
| | 2 | 465.72 | 0.07587 |
| | 3 | 200.67 | 0.05989 |
| 0.32 | 1 | 118.90 | 0.04978 |
| | 2 | 87.44 | 0.04406 |
| | 3 | 79.06 | 0.04222 |
| 0.56 | 1 | 0.00 | 0.00000 |
| | 2 | 0.00 | 0.00000 |
| | 3 | 0.00 | 0.00000 |

# APPENDIX II

## STATISTICS: CELL GROWTH (0-48 HOURS)

Chi-square test for normality: actual and expected frequencies

| INTERVAL | <-1.5 | -1.5 to <-0.5 | -0.5 to 0.5 | >0.5 to 1.5 | >1.5 |
|---|---|---|---|---|---|
| EXPECTED | 1.541 | 5.566 | 8.786 | 5.566 | 1.541 |
| OBSERVED | 1 | 6 | 9 | 7 | 0 |

Calculated Chi-Square goodness of fit test statistic = 2.1394
Table Chi-Square value (alpha = 0.01) = 13.277
Data PASS normality test. Continue analysis.

Bartletts test for homogeneity of variance

Calculated B statistic = 11.31
Table Chi-square value = 16.81 (alpha = 0.01)
Table Chi-square value = 12.59 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 2.29
Used for Chi-square table value ==> df (#groups-1) = 6

Data PASS homogeneity test at 0.01 level. Continue analysis.
NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

| GROUP | IDENTIFICATION | N | ORIGINAL MEAN | TRANSFORMED MEAN | ISOTONIZED MEAN |
|---|---|---|---|---|---|
| 1 | Control | 6 | 1097.898 | 1097.898 | 1206.645 |
| 2 | 0.018 mg/l | 2 | 1349.510 | 1349.510 | 1206.645 |
| 3 | 0.032 mg/l | 3 | 1328.893 | 1328.893 | 1206.645 |
| 4 | 0.056 mg/l | 3 | 1161.847 | 1161.847 | 1161.847 |
| 5 | 0.10 mg/l | 3 | 924.217 | 924.217 | 924.217 |
| 6 | 0.18 mg/l | 3 | 390.650 | 390.650 | 390.650 |
| 7 | 0.32 mg/l | 3 | 95.133 | 95.133 | 95.133 |

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

| IDENTIFICATION | ISOTONIZED MEAN | CALC. WILLIAMS | SIG P=.05 | TABLE WILLIAMS | DEGREES OF FREEDOM |
|---|---|---|---|---|---|
| Control | 1206.645 | | | | |
| 0.018 mg/l | 1206.645 | 0.787 | | 1.75 | k= 1, v=16 |
| 0.032 mg/l | 1206.645 | 0.908 | | 1.83 | k= 2, v=16 |
| 0.056 mg/l | 1161.847 | 0.534 | | 1.86 | k= 3, v=16 |
| 0.10 mg/l | 924.217 | 1.451 | | 1.87 | k= 4, v=16 |
| 0.18 mg/l | 390.650 | 5.907 | * | 1.88 | k= 5, v=16 |
| 0.32 mg/l | 95.133 | 8.375 | * | 1.89 | k= 6, v=16 |

$ = 169.334
Note: df used for table values are approximate when v > 20.

# APPENDIX II

## STATISTICS: GROWTH RATE (0-48 HOURS)

Chi-square test for normality: actual and expected frequencies

| INTERVAL | <-1.5 | -1.5 to <-0.5 | -0.5 to 0.5 | >0.5 to 1.5 | >1.5 |
|---|---|---|---|---|---|
| EXPECTED | 1.541 | 5.566 | 8.786 | 5.944 | 1.963 |
| OBSERVED | 1 | 6 | 8 | 8 | 0 |

Calculated Chi-Square goodness of fit test statistic = 2.8993
Table Chi-Square value (alpha = 0.01) = 15.277
Data PASS normality test. Continue analysis.

Hartley test for homogeneity of variance

Calculated H statistic (max Var/min Var) = 382.95
Closest, conservative, Table H statistic = 1705.0 (alpha = 0.01)

Used for Table H ==> R (# groups) = 7, df (# reps-1) = 2
Actual values ==> R (# groups) = 7, df (# avg reps-1) = 2.29
(average df used)

Data PASS homogeneity test. Continue analysis.
NOTE: This test requires equal replicate sizes. If they are unequal but do not differ greatly, the Hartley test may still be used as an approximate test (average df are used).

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

| GROUP | IDENTIFICATION | N | ORIGINAL MEAN | TRANSFORMED MEAN | ISOTONIZED MEAN |
|---|---|---|---|---|---|
| 1 | Control | 6 | 0.091 | 0.091 | 0.092 |
| 2 | 0.018 mg/l | 2 | 0.094 | 0.094 | 0.092 |
| 3 | 0.032 mg/l | 3 | 0.094 | 0.094 | 0.092 |
| 4 | 0.056 mg/l | 3 | 0.091 | 0.091 | 0.091 |
| 5 | 0.10 mg/l | 3 | 0.088 | 0.088 | 0.088 |
| 6 | 0.18 mg/l | 3 | 0.071 | 0.071 | 0.071 |
| 7 | 0.32 mg/l | 3 | 0.045 | 0.045 | 0.045 |

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

| IDENTIFICATION | ISOTONIZED MEAN | CALC. WILLIAMS | SIG P=.05 | TABLE WILLIAMS | DEGREES OF FREEDOM |
|---|---|---|---|---|---|
| Control | 0.092 | | | | |
| 0.018 mg/l | 0.092 | 0.346 | | 1.75 | 6, 1, 0.10 |
| 0.032 mg/l | 0.092 | 0.400 | | 1.83 | 6, 2, 0.10 |
| 0.056 mg/l | 0.091 | 0.136 | | 1.86 | 6, 3, 0.10 |
| 0.10 mg/l | 0.088 | 0.667 | | 1.87 | 6, 4, 0.10 |
| 0.18 mg/l | 0.071 | 5.105 | * | 1.88 | 6, 5, 0.10 |
| 0.32 mg/l | 0.045 | 11.698 | * | 1.89 | 6, 6, 0.10 |

t = 0.003
Note: df used for table values are approximate when v > 20.